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Diabetes-induced alterations in tissue collagen and carboxymethyllysine in rat kidneys: Association with increased collagen-degrading proteinases and amelioration by Cu(II)-selective chelation

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ABSTRACT

Advanced glycation end-products (AGEs) comprise a group of non-enzymatic post-translational modifications of proteins and are elevated in diabetic tissues. AGE-modification impairs the digestibility of collagen *in vitro* but little is known about its relation to collagen-degrading proteinases *in vivo*. N^{ε}-carboxymethyllysine (CML) is a stable AGE that forms on lysyl side-chains in the presence of glucose, probably *via* a transition metal-catalysed mechanism.

Here, rats with streptozotocin-induced diabetes and non-diabetic controls were treated for 8 weeks with placebo or the Cu(II)-selective chelator, triethylenetetramine (TETA), commencing 8 weeks after disease induction. Actions of diabetes and drug treatment were measured on collagen and collagen-degrading proteinases in kidney tissue.

The digestibility and CML content of collagen, and corresponding levels of mRNAs and collagen, were related to changes in collagen-degrading-proteinases. Collagen-degrading proteinases, cathepsin L (CTSL) and matrix metalloproteinase-2 (MMP-2) were increased in diabetic rats. CTSL-levels correlated strongly and positively with increased collagen-CML levels and inversely with decreased collagen digestibility in diabetes. The collagen-rich mesangium displayed a strong increase of CTSL in diabetes. TETA treatment normalised kidney collagen content and partially normalised levels of CML and CTSL.

These data provide evidence for an adaptive proteinase response in diabetic kidneys, affected by excessive collagen-CML formation and decreased collagen digestibility. The normalisation of collagen and partial normalisation of CML- and CTSL-levels by TETA treatment supports the involvement of Cu(II) in CML formation and altered collagen metabolism in diabetic kidneys. Cu(II)-chelation by TETA may represent a treatment option to rectify collagen metabolism in diabetes independent of alterations in blood glucose levels.

1. Introduction

CML is a major AGE that is said to form *via* the 'glycoxidation pathway' (also known as 'autoxidative glycation'), which involves the reaction of glucose with proteins, catalysed by transition metals such as copper [1,2]. The AGE formation is enhanced in diabetes [3] where it has been linked to decreased collagen digestibility [4,5] and altered proteolytic processing of albumin *in vitro* [6].

The capacity of the proteolytic system may eventually become exhausted by excessive AGE formation and AGE accumulation in lysosomes has been demonstrated *in vitro* [7]. Consequently, proteinases involved in the degradation of collagen could be affected by altered AGE content of substrate proteins in diabetes, but data concerning *in vivo* collagen-AGE formation that pertain to roles of collagen-degrading proteinases are lacking. Distinguishing between substrates modified with AGEs *in vitro* compared with *in vivo* is recognised as an important question, and has generated discussion regarding the interaction of AGE-modified proteins with the receptor for AGEs (RAGE) [8].

MMP-2 and certain cathepsins are implicated in soft-tissue collagen turnover [9,10]. Previous reports regarding collagen degradation in diabetic kidneys are contradictory. Increased proteolytic activity towards

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glomerular basement-membrane collagen has been reported in glomerular homogenates from rats with streptozotocin (STZ)-induced diabetes after 4 and 10 weeks' hyperglycaemia [11]. This finding contrasts with data obtained using artificial substrates for collagen-degrading cathepsins as well as gelatine zymography for metalloproteinases, whereby decreased activity was reported in glomeruli of STZ-diabetic rats with up to 5-weeks' diabetes [12]. Activity of CTSL was reportedly decreased in kidneys of STZ-diabetic rats 10 and 30 days after disease induction [13] while activity was decreased and unchanged after 10 days and 6-months in proximal-tubular cells, respectively [14].

The aim of this study was to investigate the relation between modifications of collagen and collagen-degrading proteinases in healthy and STZ-diabetic rats. The Cu(II) chelator TETA, was previously shown to prevent albuminuria and heart failure in diabetic rats [15,16], and was here investigated as an interventional treatment against AGE formation and linked effects on collagen homeostasis.

2. Materials and methods

2.1. Animal studies

All protocols were approved by the University of Auckland Animal Ethics Committee and complied with the 'Principles of Laboratory Animal Care' (NIH 1985): this report is consistent with current guidelines for the proper reporting of animal experiments [17]. Male Wistar rats 8 weeks of age with body-weights of 300 g (±30 g) (mean ± SEM) were injected once *via* the tail vein with a solution of saline vehicle or STZ (S0130, Lot No. 119K1591, Sigma-Aldrich, USA; 55.0 mg/kg body-weight) in saline solution. Rats were fed chow (Teklad Diet No. 2018; Harlan Laboratories, USA) and water *ad libitum*. Thereafter, body-weights of animals were measured weekly and non-fasting blood-glucose levels fortnightly. Treatment of rats with placebo (water) or TETA dihydrochloride (~17 mg/day; T5033, Sigma-Aldrich, USA) was started 8 weeks after injection in one-half of the STZ- and saline-injected animals, and was administered *via* the drinking water as previously described [15]. Kidneys were perfused and collected at the end of experimental week 16, following 8-weeks' drug treatment. RNAlater (AM7021, Life Technologies, USA) was added to those samples destined for RNA analysis and samples were stored at −80 °C. Light microscopy of haematoxylin-and-eosin-stained renal-cortical sections was performed, and showed that rats had not developed renal tumours, consistent with a lack of renal STZ toxicity.

2.2. Gene expression analysis

RNA was extracted from renal cortical tissue using spin columns (QIAGEN, Germany). Complementary DNA synthesised using the Transcriptor cDNA kit with an oligo-dT primer (Roche, Germany) and subsequently analysed using the Light Cycler 480 system and SYBR green MasterMix (both Roche). Target gene levels were normalised to three reference genes, which were stably and similarly expressed in all four groups of animals. All primers (Table 1) were designed to be intron-spanning.

Table 1
Primer sequences for RT qPCR.

List of primers		
Gene abbreviation	Forward primer sequence	Reverse primer sequence
Col1a1	ACATGTTCAGCTTTGTGGACC	TTAGGGACCCTTAGGCCATT
Col1a2	AGAAAATGGCATCGTTGGTC	ACCAGGGAAGCCAGTCATAC
Col3a1	TATTTTGGCACAGCAGTCCA	CAGAGGACAGATCCCGAGTC
Col4a1	GTCCTCACTGTGGATTGGCT	CGATGAATGGGGCACTTCTA
Col4a4	CAGATGGACCGAGTCCTACG	TCTTGCTGCTCCCATATTCA
Col4a5	GATCTCCAGGTGACCAAGGA	CCTGAAATGCCAGTTCCAAT
Col6a1	GCTGAGCAAGGACGAGCTAG	CACGTGTTCTTGCATCTGGT
Col6a2	GACTTTGGTCTGAAAGGAGCA	GGGTTCTCCCTCAGGTCCT
Col6a3	TACCGAGCATCTGAGGAGCT	GAACTCCACTTGCATCAGCC
Ctsl	AAGCCCTCATGAAGCCTGTAG	CCTTGCTGCTACAGTTGGGTT
Lox	CTGACTTCTTACCAAGCCGC	GCAGGTCGTAGTGGCTGAAT
Mmp2	CTTTGATGGCATTGCTCAGA	GTCACGTGGTGTCACTGTCC
Rpl13a	ACAAGAAAAAGCGGATGGTG	TTCCGGTAATGGATCTTTGC
Tbp	AGAACAATCCAGACTAGCAGCA	GGGAACTTCACATCACAGCTC
U2af	CCATTGCCCTCTTGAACATT	CCTCCCCGTACTTCTCTTCC

2.3. Western blotting

Protein levels of lysyl oxidase (LOX) (sc-32410; Santa Cruz, USA) and CTSL (ab6314; Abcam, UK) in kidney-cortex lysates were detected by Western blotting as described [16], and normalised to corresponding beta-actin values (sc-47778; Santa Cruz).

2.4. Gelatinase activity assay

Tissue samples were lysed (10 mM $CaCl_2$, 0.25% v/v Triton-X100) and run on gelatine-containing polyacrylamide gels (EC 61752, Life Technologies, USA). These were equilibrated at RT for 30 min in renaturation buffer (LC2670, Life Technologies), and then incubated in developing buffer (LC2671, Life Technologies) for 30 min at RT. Buffer was then refreshed, and gels incubated for 72 h at 37 °C. Gels were stained with Coomassie blue [18]. EDTA (10 mM) was added as a negative control to the first incubations on a separate control gel to confirm that the activity was metalloproteinase-dependent.

2.5. CTSL activity assay

CTSL activity was assayed using a peptide substrate, Ac-HRYR-ACC (Cat # 219497; Merck, Germany), coupled to the probe, ACC (7-amino-4-carbamoylmethylcoumarin), which fluoresces following cleavage. The assay was performed as described by Choe et al. [19] with minor modifications. In brief, renal cortex was lysed (30 mM Tris–HCl, 0.1% Brij-35 v/v, 1 mM DTT) and mixtures centrifuged (15 min, 16,100 *g*, 4 °C). Supernatants were collected on ice, and 10 μL (~30 μg) of each was added in triplicate to a black 96-well plate. The activity-assay buffer (100 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, 0.01% Brij-35 (v/v), DTT 10 mM, pH 5.5 with substrate/DMSO to 5 μM) was pre-warmed to 30 °C, and added alongside a standard curve. Fluorescence was measured (excitation, 380 nm; emission, 460 nm; cut-off, 435 nm) after 10 min incubation at 30 °C. Activity was normalised for protein concentration as determined by BCA assay. TETA (25 μM) and/or $CuCl_2$ (25 μM) were added for relative CTSL activity assay, and comparisons made to wells containing the lysate without either of these compounds (100% activity).

2.6. Collagen extraction

Collagen was extracted from kidney tissues after removal of the renal pelvis and major calyces, and purified according to a published protocol [20].

2.7. Quantification of collagen levels

Collagen was quantitated by measurement of hydroxyproline levels in acid hydrolysates (6 M HCl at 105 °C for 16 h) of the kidney or collagen extracts according to the procedure of Bergman and Loxley [21].

2.8. Determination of collagen pepsin digestibility

Collagen extracts were digested with 1 mg/ml pepsin/0.5 M acetic acid at a concentration of 1 mg/ml for 24 h at 4 °C. Digests were then centrifuged (16,100 *g*, 4 °C, 30 min). Hydroxyproline levels in supernatants and pellets were measured, and the digestibility presented as the percentage of total collagen.

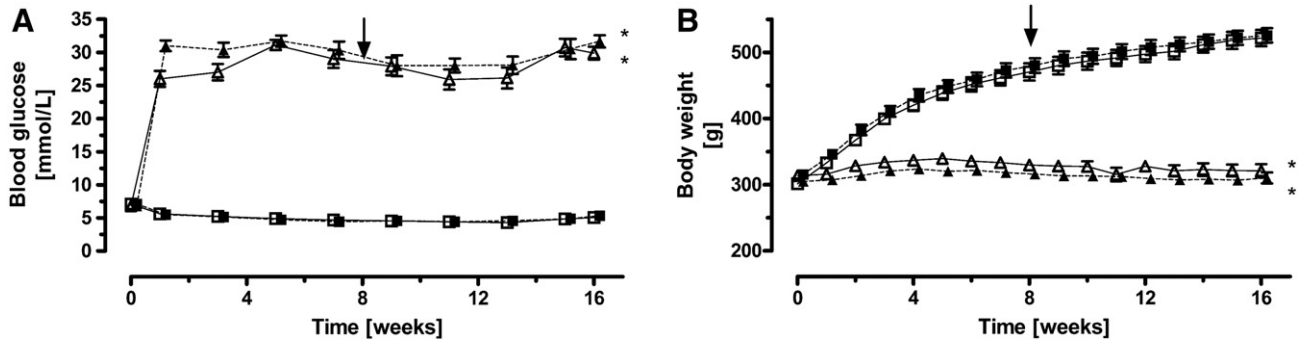

Fig. 1. Average blood-glucose levels (A) and body-weights (B) of the four groups over 16 weeks are shown. Commencement of placebo or TETA treatment is as indicated (black arrow). Symbols are: white squares, Sham/Plac; black squares, Sham/TETA; white triangles, Dia/Plac; black triangles, Dia/TETA: $n = 11/12$ per group; and *, $P < 0.05$ *vs.* Sham/Plac. *Nonstandard abbreviations*: Dia, diabetic; Plac, placebo-treated; and TETA, triethylenetetramine-treated.

2.9. LC MS/MS analysis

Collagen extracts were solubilised by digestion with pepsin and separated by SDS-PAGE, stained with Coomassie blue [18]. Individual bands were de-stained and proteins identified using a QSTAR-XL Quadrupole Time-of-Flight mass spectrometer (Applied Biosystems, USA) following in-gel trypsin digestion.

2.10. Quantification of CML levels

CML levels were measured in kidney collagen extracts solubilised with Liberase DH (Roche, Germany). Equal amounts of collagen, as determined by hydroxyproline measurement, were loaded in triplicate onto a nitrocellulose membrane. An AGE standard (produced by incubating BSA with fructose/PBS for 8 weeks followed by dialysis) was loaded onto each blot for purposes of normalisation. CML levels were detected using a monoclonal antibody (KAL-KH024, Cosmo Bio, Japan) followed by an HRP-conjugated secondary antibody and ECL detection, after which ECL signals were quantitated by densitometry. Specificity of the antibody was confirmed by treatment with the CML-blocking peptide (YAAA(CML)AAA), which was synthesised in-house as described previously [22].

2.11. Immunofluorescent staining

Staining was performed as described previously [23] with minor modifications. In short, frozen sections were acetone-fixed and stained using primary antibodies against CTSL (ab6314, Abcam, UK) and type 1 collagen (ab34719, Abcam, UK), and Alexa 647- (A21240, Life Technologies, USA) and Alexa 488-coupled (A11034, Life Technologies, USA) secondary antibodies, respectively. Sections were treated with Sudan Black B (199664, Sigma-Aldrich, USA) [24] prior to fixation and DAPI counterstaining (P36941, Life Technologies, USA). Sections were analysed using a fluorescence microscope (Leica DMR, Leica, Germany) and camera (Spot pursuit, Spot Imaging Solutions, USA).

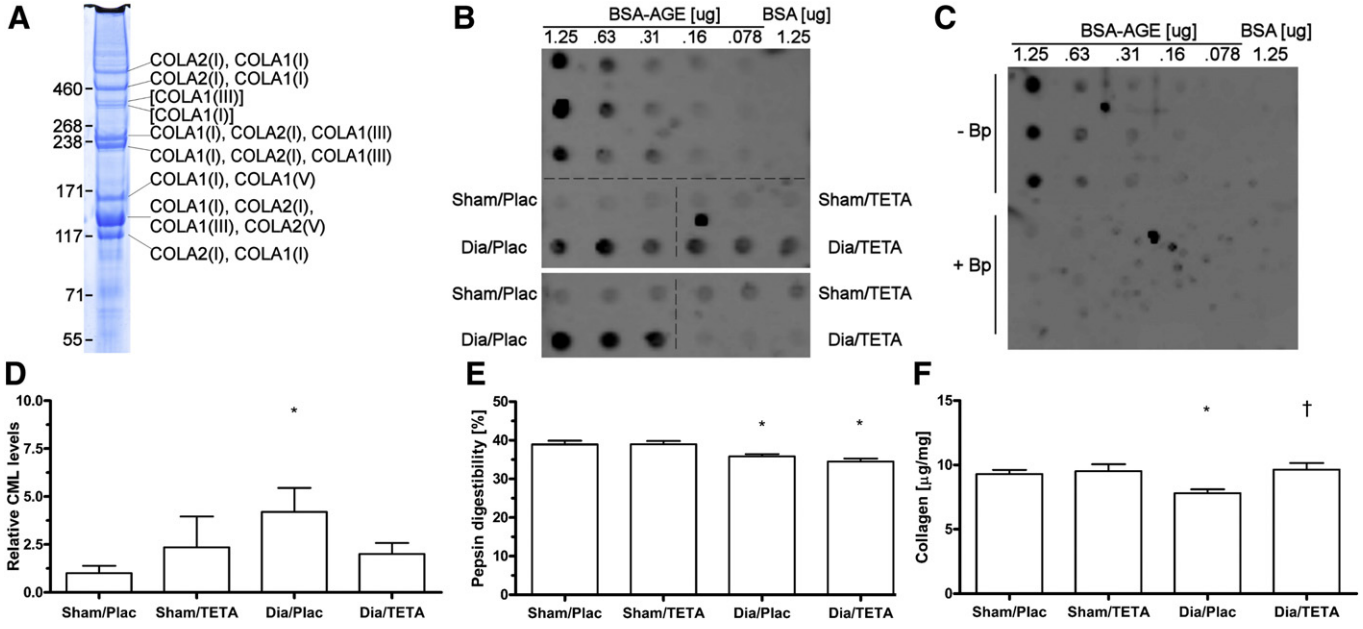

Fig. 2. Annotated collagen isoforms on Coomassie blue-stained SDS-PAGE (A) were identified by LC MS/MS analysis or by comparison to the available literature (*shown in parentheses*). Molecular weight marker [kD] is indicated. A representative blot for the quantification of collagen-bound CML including a BSA-AGE standard curve is shown in (B) with triplicates shown for each sample. Specificity of the signal was confirmed in (C) by incubation of the monoclonal antibody with (+BP) or without a CML-blocking peptide (-BP). Quantification of CML levels (D) and pepsin digestibility (E) of kidney collagen extracts of the four groups are shown alongside collagen levels in kidney cortices (F) of the groups: $n = 11/12$ per group: *, $P < 0.05$ *vs.* Sham/Plac; †, $P < 0.05$ *vs.* Dia/Plac. *Nonstandard abbreviations*: AGE, advanced glycation end-product; Bp, blocking peptide; BSA, bovine serum albumin; CML, N^{ε}-carboxymethyllysine; Dia, diabetic; LC, liquid chromatography; MS, mass spectrometry; and Plac, placebo-treated.

Table 2
Relative mRNA levels of the main collagen isoforms were measured in the kidney cortex of the four groups.

Gene abbreviation	Sham/Plac	Sham/TETA	Dia/Plac	Dia/TETA
Col1a1	1.00 ± 0.21	0.93 ± 0.11	0.90 ± 0.09	1.09 ± 0.23
Col1a2	1.00 ± 0.12	0.95 ± 0.09	0.61 ± 0.07	0.64 ± 0.13*
Col3a1	1.00 ± 0.22	1.24 ± 0.15	0.75 ± 0.09	0.75 ± 0.34
Col4a1	1.00 ± 0.08	1.20 ± 0.13	0.82 ± 0.08	0.98 ± 0.12
Col4a4	1.00 ± 0.10	0.95 ± 0.06	0.39 ± 0.05*	0.51 ± 0.07*
Col4a5	1.00 ± 0.06	0.81 ± 0.07	0.68 ± 0.05*	0.64 ± 0.03*
Col6a1	1.00 ± 0.10	1.03 ± 0.12	0.62 ± 0.10*	0.64 ± 0.12
Col6a2	1.00 ± 0.10	0.91 ± 0.10	0.59 ± 0.08*	0.57 ± 0.09*
Col6a3	1.00 ± 0.08	1.36 ± 0.15	0.78 ± 0.08	0.93 ± 0.24

Values are mean ± SEM and presented as relative to the Sham/Plac group: $n = 8/9$ per group.
* $P < 0.05$ *vs.* Sham/Plac.

2.12. Statistical analysis

Data were analysed by two-way ANOVA followed by Tukey–Kramer *post-hoc* tests (for normally-distributed data), repeated-measures ANOVA, or the Mann–Whitney U test (for non-parametric data), as appropriate. All data are shown as mean ± SEM, and differences have been considered significant at $P < 0.05$. All between-subject variability of measurements has been retained in all analyses: no 'outlying' values were removed from any of the data sets.

3. Results

3.1. Disturbed collagen equilibrium was associated with changes in post-translational modification in diabetic kidneys: amelioration by TETA treatment

STZ-injected rats became diabetic within 2 days and displayed high blood-glucose levels throughout the remainder of the study (Fig. 1 A). Diabetic rats had decreased body-weights starting from week 2 (Fig. 1 B). Treatment with TETA influenced neither blood-glucose levels nor body-weight in non-diabetic or diabetic rats.

Kidney collagen extracts from healthy animals were solubilised by pepsin treatment and qualitatively characterised by LC MS/MS analysis of proteins separated using Coomassie blue-stained SDS-PAGE gels (Fig. 2 A). Type I collagen was identified as the major isoform, along with lesser amounts of collagen types III and V: these findings are consistent with other available data [25,26].

A representative blot for the measurement of collagen-bound CML levels from two animals per group (Fig. 2 B) and the blocking of the BSA-AGE signal after incubation of the antibody with a CML-blocking peptide, are shown (Fig. 2 C). CML levels were significantly increased in renal collagen extracts from diabetic rats (Fig. 2 D). Eight weeks' TETA treatment decreased collagen-bound CML content to levels not significantly different from those in the healthy placebo-treated group. Pepsin digestibility of collagen extracts in diabetic tissue was decreased and TETA treatment was without effect in either control or diabetic tissue (Fig. 2 E). Collagen levels, as determined by hydroxyproline measurement, were decreased in the renal cortex of diabetic rats and normalised by TETA treatment (Fig. 2 F). The decrease in collagen levels in diabetes was accompanied by a trend towards decrease in mRNA levels corresponding to all major isoforms of collagen that we detected in kidney tissue (Table 2). This effect was significant for four of the isoforms whereas TETA treatment had no effect on collagen mRNA levels. Messenger RNA and protein levels of LOX were increased in the kidney cortex of diabetic rats (Fig. 3 A–C).

3.2. Increased levels of collagen-degrading proteinases in kidneys of diabetic rats

Mmp2 mRNA levels showed a tendency towards increase in diabetic rats whereas TETA treatment was without effect (Fig. 4 A). Kidney cortex lysates were analysed for metalloproteinase activity by gelatine zymography (Fig. 4 B) and quantified by densitometry measurement (Fig. 4 C and D). Overall activity levels for pro-MMP2 (70 kDa) where much higher than those of MMP-2 (60 kDa): this is likely due to the presence of inhibitors which bind to activated MMP-2 [27]. MMP-2 activity levels displayed a tendency towards increase in the placebo- but not the TETA-treated diabetic group (Fig. 4 C). Activity levels of pro-MMP-2 were significantly increased in diabetes (Fig. 4 D) whereas a trend towards decreased MMP-2 activity levels was seen in TETA-treated diabetic animals. *Ctsl* mRNA levels were increased in diabetic renal cortices (Fig. 5 A). CTSL single-chain (CTSL sc) as well as the processed heavy-chain of CTSL double-chain (CTSL dc) were detected by Western blotting (Fig. 5 B) [28]. Importantly both CTSL sc and CTSL dc display activity [29]. Levels were quantified and CTSL sc (Fig. 5 C) and CTSL dc (Fig. 5 D) were increased in diabetic renal cortices whereas TETA treatment displayed a tendency to decrease CTSL dc levels. CTSL activity was also slightly increased in diabetic rats (Fig. 5 E). Addition of $CuCl_2$ *in vitro* decreased CTSL activity in kidney lysates from placebo-treated healthy and diabetic rats (Fig. 5 F) whereas the presence of TETA normalised activity in lysates from both groups.

3.3. Increased CTSL levels in the collagen-rich mesangium and interstitium as well as tubule cells of diabetic rats and amelioration by TETA treatment

Type 1 collagen (COL1, Fig. 6 A–C) and CTSL (Fig. 6 D–F) were detected by immunofluorescence staining in kidney cortices of placebo-treated healthy, and placebo-treated and TETA-treated diabetic rats. Nuclei were stained with DAPI (Fig. 6 G–I) and overlay images with immunofluorescent staining are shown (Fig. 6 J–L). In line with the results of quantitative collagen measurement by hydroxyproline assay, collagen staining was slightly weaker in placebo-treated diabetic rats (Fig. 6 B) compared to placebo-treated healthy rats (Fig. 6 A) while staining in TETA-treated diabetic rats (Fig. 6 C) was slightly stronger. CTSL in placebo-treated diabetic *vs.* healthy rats (Fig. 6 D *vs.* E) was strongly increased. The increase was most pronounced in collagen-

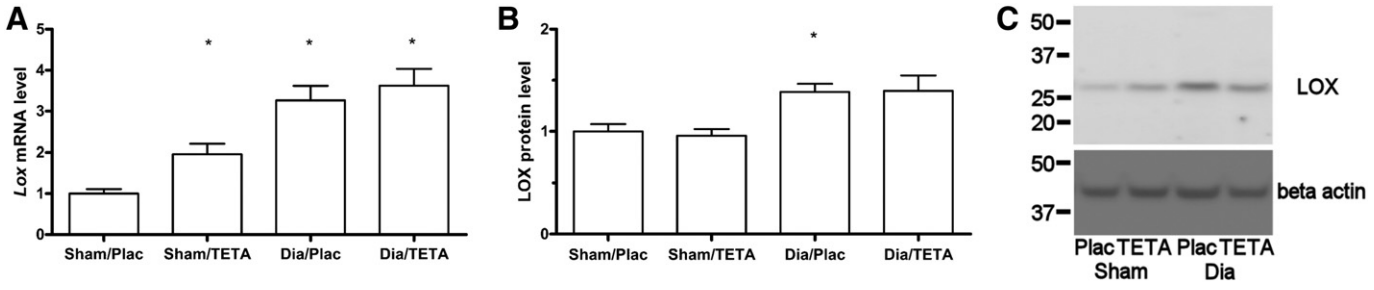

Fig. 3. Relative *Lox* mRNA levels (A), LOX protein levels (B) and a representative Western blot (C), are shown for renal-cortical tissue of the four treatment groups. $n = 18/19$ per group: *, $P < 0.05$ *vs.* Sham/Plac. *Nonstandard abbreviations*: LOX, lysyl oxidase; Plac, placebo-treated.

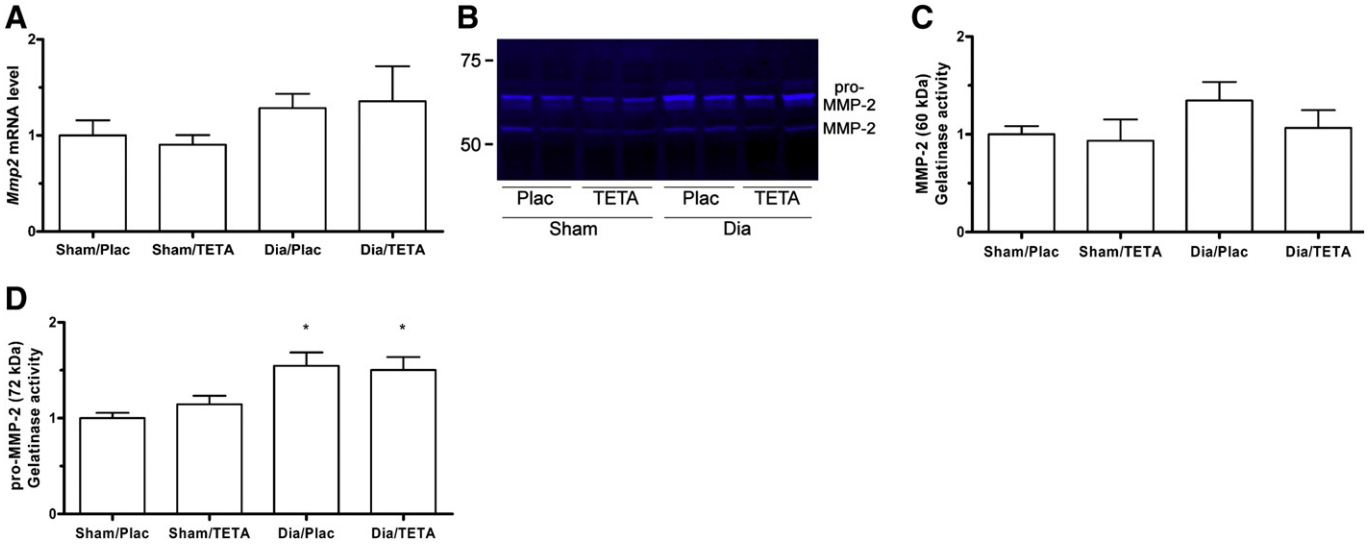

Fig. 4. *Mmp2* mRNA levels in kidney cortices from the four treatment groups ($n = 8/9$ per group) are shown in (A). A representative zymography gel for kidney cortices of the four groups is shown in (B). Quantification of activity for the four groups ($n = 11/12$ per group) at the molecular masses of 60 kDa for MMP-2 (C) and 72 kDa for pro-MMP-2 (D) are shown: *, $P < 0.05$ *vs.* Sham/Plac. *Nonstandard abbreviations*: Mmp, matrix metalloproteinase; Plac, placebo-treated.

rich areas of the mesangium and the peritubular interstitium as well as the apex of tubular cells. Treatment of diabetic rats with TETA (Fig. 6 F) reversed the increase of CTSL. At higher magnification (Fig. 7 A–L) staining of COL1 is seen to be stronger in placebo-treated healthy animals (Fig. 7 A) compared to placebo-treated diabetic animals (Fig. 7 B) with amelioration in TETA-treated diabetic animals (Fig. 7 C). Strong staining for CTSL in placebo-treated diabetic animals compared to healthy animals (Fig. 7 D and E) is also present. CTSL staining is pronounced in the proximity of COL1 staining (Fig. 7 B and K) in the glomerulus as well as in the surrounding peritubular interstitium while CTSL staining is weaker in TETA-treated diabetic animals (Fig. 7 F).

3.4. CTSL expression correlates strongly with extent of collagen modifications

A strong positive correlation between CTSL sc and -dc levels and collagen CML levels was present (Fig. 8 A and B). CTSL sc and -dc levels were inversely correlated with pepsin digestibility (Fig. 8 C and D) in non-diabetic and diabetic placebo treated animals. Collagen levels displayed an inverse correlation with pro-MMP-2 (Fig. 8 E) in these animals.

4. Discussion and conclusion

The formation of AGEs in general and CML in particular is enhanced in diabetes [3], and AGE formation impairs the digestibility of affected proteins *in vitro* [4] providing a potential link with collagen accumulation and fibrosis [30]. Here, a decrease in the digestibility of collagen from kidneys of diabetic animals was paralleled by elevated collagen CML levels without collagen accumulation. On the contrary a decrease of collagen levels was present in diabetic animals.

The diabetic state modifies collagen-degrading proteinases [11,31] which may alter collagen levels. Here, the collagen-degrading lysosomal proteinase CTSL and the extracellular proteinase MMP-2 were increased

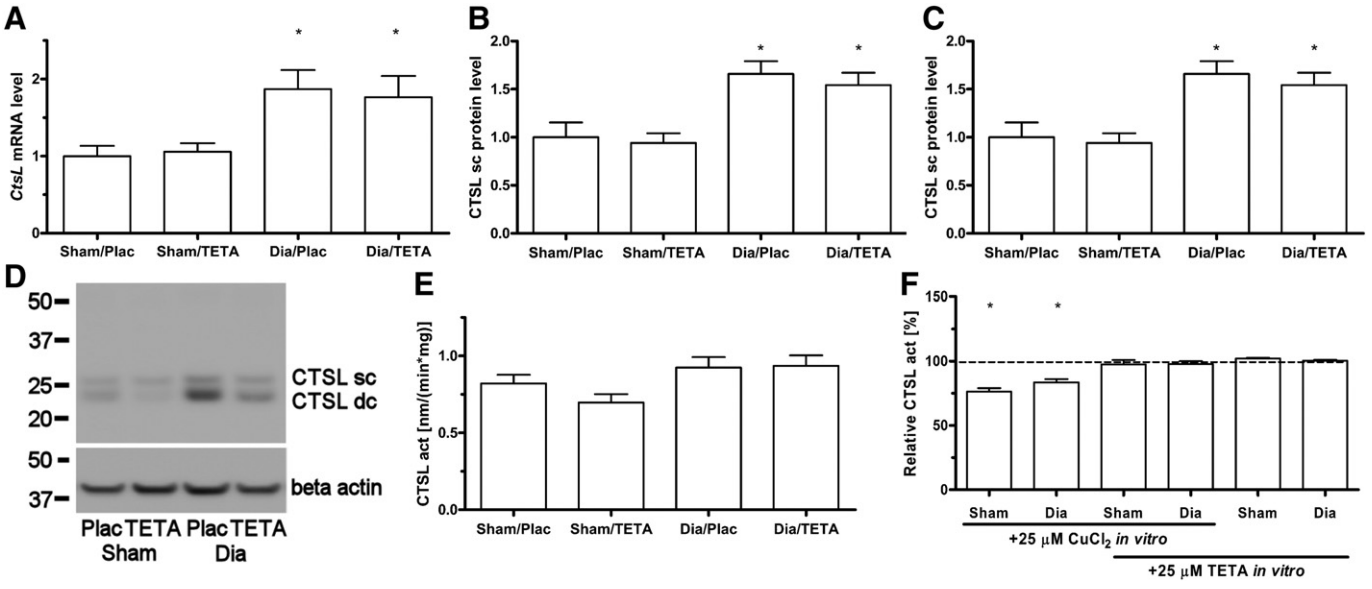

Fig. 5. *Ctsl* mRNA levels (A, $n = 8/9$ per group) and protein levels for CTSL-sc (B) and CTSL-dc (C) in kidney cortices of the four groups ($n = 11/12$ per group) are shown alongside a representative Western blot (D). CTSL activity measurement ($n = 11/12$ per group) is shown (E). Effect of *in vitro* addition of $CuCl_2$ and TETA on CTSL activity in placebo-treated healthy and diabetic kidneys is shown relative to control (F). *, $P < 0.05$ *vs.* Sham/Plac. *Nonstandard abbreviations*: act, enzyme activity; CTSL, cathepsin L; dc, double-chain; Dia, diabetic; Plac, placebo-treated; sc, single-chain; and TETA, triethylenetetramine.

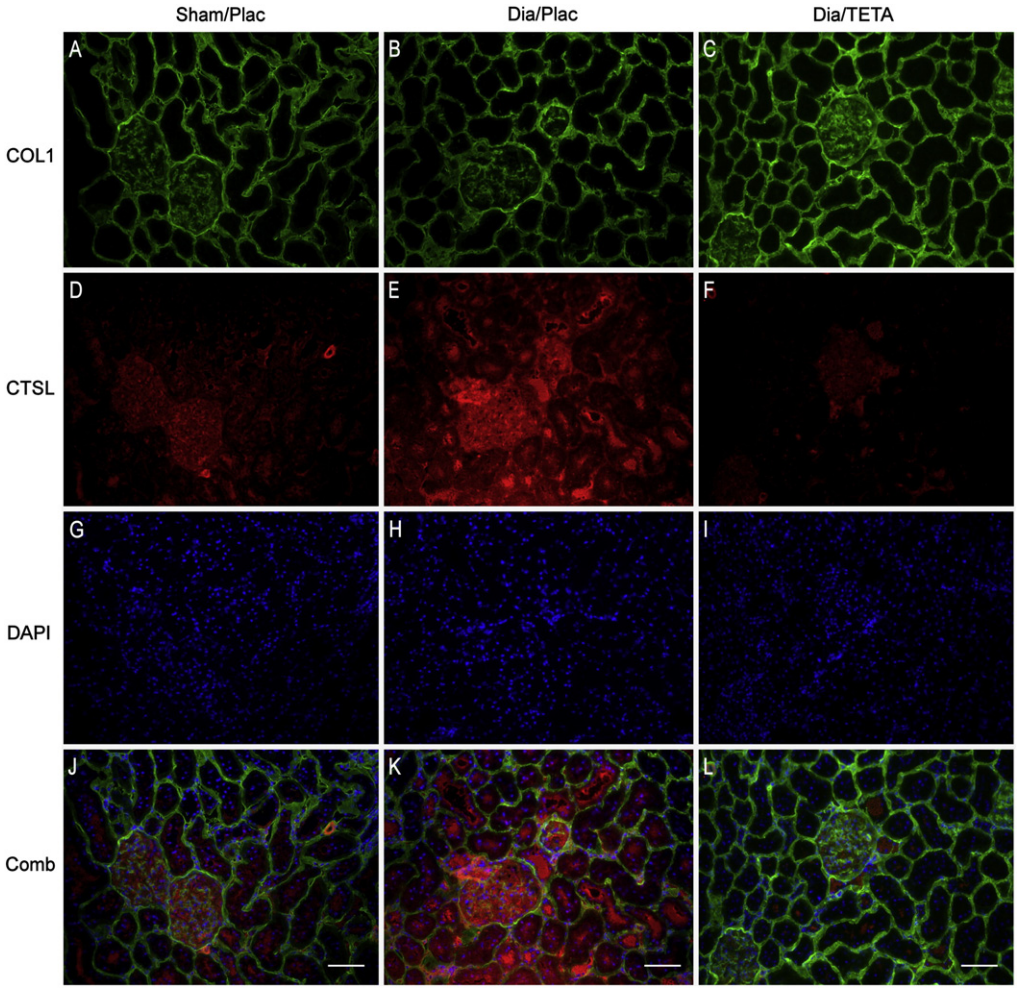

Fig. 6. Immunofluorescence staining of kidney cortex with groups indicated at the top and staining on the side of images, respectively. Sections are stained with type 1 collagen- (COL1) and cathepsin L (CTSL) specific primary antibodies and Alexa Fluor 488 or Alexa Fluor 647 coupled secondary antibodies, respectively. Nuclei are stained with DAPI. An image overlay (Comb) of the channels is depicted in the bottom row (scale bar = 100 μm).

in diabetic animals. Activity levels of the latter correlated inversely with collagen levels, supporting the importance of this protease for collagen degradation. Furthermore, levels of the lysosomal proteinase CTSL, which acts downstream of MMP-2 [9], correlated strongly with the levels of the collagen-AGE adduct CML and inversely with pepsin digestibility of the collagen extracts. Thus, the data presented here provide the first *in vivo* evidence for a connection between a collagen-bound AGE and a possibly adaptive dysregulation of the lysosomal proteinase CTSL.

This concept is supported by our finding that a marked increase of CTSL is seen in the collagen-rich renal mesangium and peritubular interstitium of diabetic rats. Peres et al. has reported decreased *Ctsl* mRNA levels 10 days after diabetes induction, whereas levels displayed a trend for increase after 4 weeks of diabetes [13]. Thus, increased *Ctsl* mRNA levels after 16 weeks of diabetes which are seen here are not in contrast to this previous report.

The increased expression of CTSL could lead to untoward effects. Elevated CTSL mRNA and protein levels have been reported in diabetic nephropathy in humans [32]. Moreover, altered CTSL expression in mice has been linked to the effacement of podocyte foot processes and generation of proteinuria [32] and shown to drive podocyte apoptosis and progression to end-stage renal disease [33]. Elevation of CTSL could also exert pathogenic effects *via* cleavage and activation of glomerular heparanase [34].

The *in vitro* proteolysis of AGE-modified substrates is impaired and a glycation-dependent, altered degradative capacity of the cell has been proposed as a cause of proteinuria [6]. The data presented here for the lysosomal proteinase CTSL provide evidence for a similar mechanism as a cause of renal fibrosis in long-standing diabetes. Lysosomes accumulate AGEs *in vitro* and the capacity of the lysosomal proteolytic system may eventually become exhausted thus leading to fibrosis [7]. The duration of the current study, which ran for 4 months, may not have been long enough for fibrosis to develop whereas increased collagen levels were reported in rats following 8 months' diabetes [35,36].

The small increase in CTSL activity as opposed to CTSL expression in diabetic rats observed in the current study might be explained by copper-mediated inhibition of CTSL activity. In support of this, the *in vitro* addition of Cu(II) inhibited CTSL activity and was normalised upon addition of TETA. The capacity to bind copper is increased for peptide-bound CML residues as compared to corresponding unmodified lysyl residues [37], and collagen-rich tendon from diabetic rats binds twice the amount of copper as that from healthy rats [38].

The partial normalisation of CML levels by TETA treatment provides evidence that collagen CML formation is at least partially due to glycoxidative stress [39] and catalysed by Cu(II) ions *in vivo*. The observed trend towards decreased CML levels rather than complete normalisation could well be explained by the slow collagen turnover rate [40,41]. TETA treatment furthermore normalised collagen levels without concomitant changes in collagen mRNA, while CTSL and MMP-2 levels displayed a trend towards normalisation in TETA-treated animals. This further supports the idea of an adaptive proteinase response in diabetic animals.

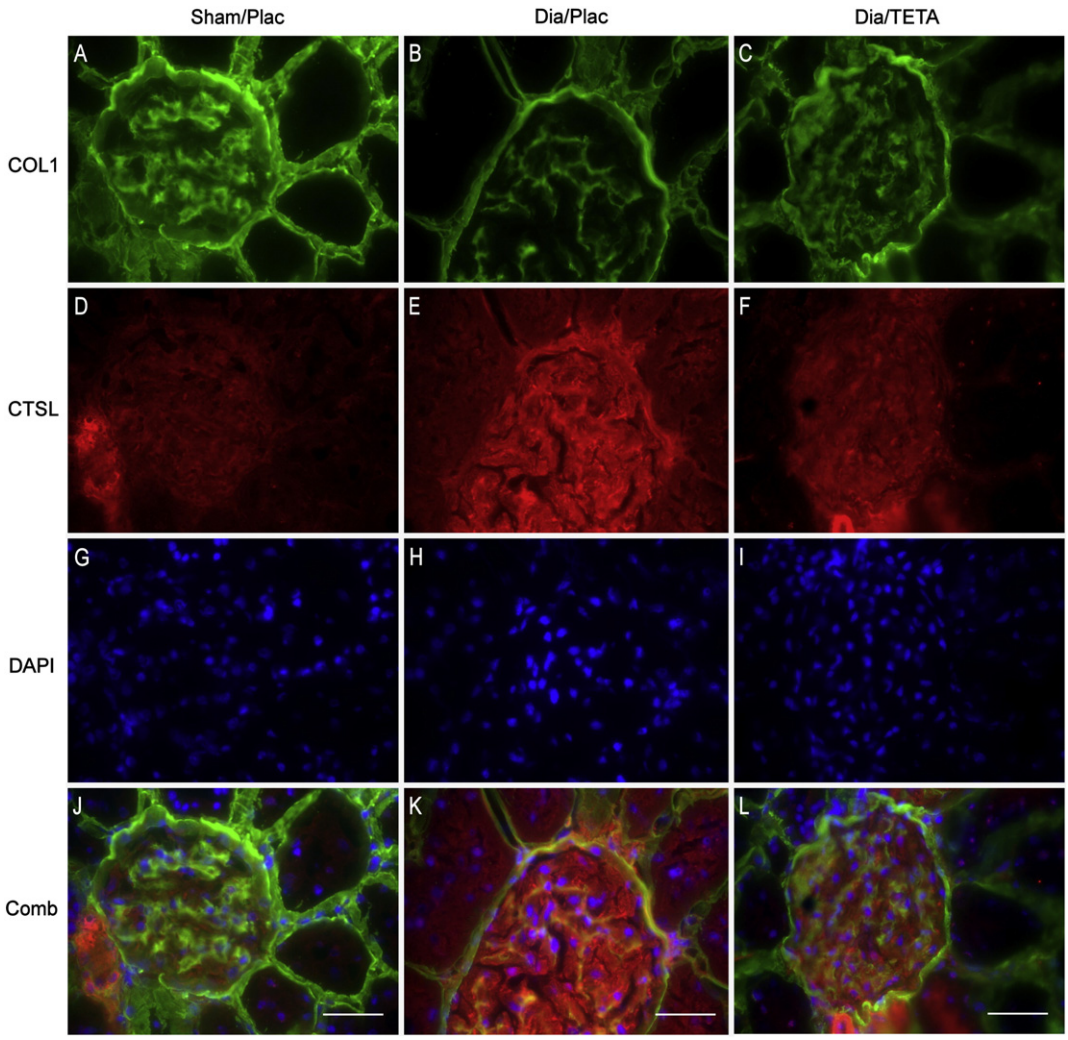

Fig. 7. Immunofluorescence staining of glomeruli (scale bar = 50 μm). Groups and staining are the same as for Fig. 6 and are indicated at the top and side of images, respectively.

Previously, decreased matrix metalloproteinase (MMP) activity has been reported after 4 days, 5 weeks [12] and 12 weeks [42] of diabetes and by using *in vitro* assays in the presence of high glucose levels [43,44]. Thus, available literature supports lower levels of MMP activity *in vitro* and at time-points early after diabetes induction, while MMP-2 levels tend to be increased after mid- to long-term diabetes according to our data. We are aware of one report of decreased MMP-2 levels, based on immunofluorescent staining of glomeruli without quantification, in a transgenic mouse model of diabetic nephropathy after 40 weeks of diabetes [45]. Differences may be due to the animal model, namely diabetes in transgenic mice *vs.* STZ-induced diabetes in rats.

LOX enzymatically cross-links collagen and may thus have an effect on the digestibility of collagen [46,47] while higher LOX levels do not necessarily increase cross-links [48]. Here, neither LOX nor collagen CML-levels correlated with collagen pepsin digestibility (correlation data not shown). Thus the decrease in collagen pepsin digestibility in diabetic animals is likely due to additive effects of a range of post-translational modifications.

How might the processes identified here be responsible for the pathological lesions of diabetic kidney disease? Skin collagen CML levels are a strong predictor for the risk of development of diabetic nephropathy in humans [49]. The data presented here shed light on the molecular basis of this association where elevated collagen CML levels and decreased collagen digestibility is paralleled by a possibly adaptive increase of the collagen-degrading lysosomal protease CTSL in kidneys from diabetic rats. The effects of AGEs on proteolysis can be manifold including altered substrate digestibility [4] and accumulation of AGEs in lysosomes *in vitro* [7]. CML in particular acts as an endogenous Cu(II) chelator [38,50]. The inhibitory effect of Cu(II) on CTSL activity and the ameliorative effect of TETA shown in the current study presents an additional mechanism for an AGE-mediated altered proteolysis.

CML bound Cu(II) may contribute to the dysregulated copper homeostasis seen in diabetes. Diabetic patients with chronic kidney disease show ~3–4-fold elevations in urinary copper output, consistent with renal copper overload [51,52]. Systemic copper overload [15,53], and elevated renal copper causes organ damage, for example in chronic dietary copper overload [54] and diabetes [16]. TETA, a Cu(II)-selective chelator [15,54,55–57], lowers elevated hepatic and CNS copper levels in Wilson's disease [58], a genetic disorder of cell-copper transport [59]. Latterly, TETA has also been applied for the experimental therapeutics of diabetes to treat defective copper homeostasis. TETA treatment here restored collagen levels and partially normalised collagen-CML levels and CTSL levels providing a link between an altered collagen metabolism and dysregulated copper metabolism in diabetes.

In summary, our results provide evidence for a pathogenic mechanism in kidneys of diabetic rats that acts through glycoxidative stress-dependent modification of collagen coupled to the dysregulation of key proteinases, specifically CTSL and MMP-2, and consequent alterations in collagen metabolism. Together, these processes may exhaust the proteolytic potential of the cell and lead to the formation of fibrosis and glomerular basement-membrane dysfunction in the kidneys of diabetic animals. We propose that chelation of Cu(II) by TETA represents

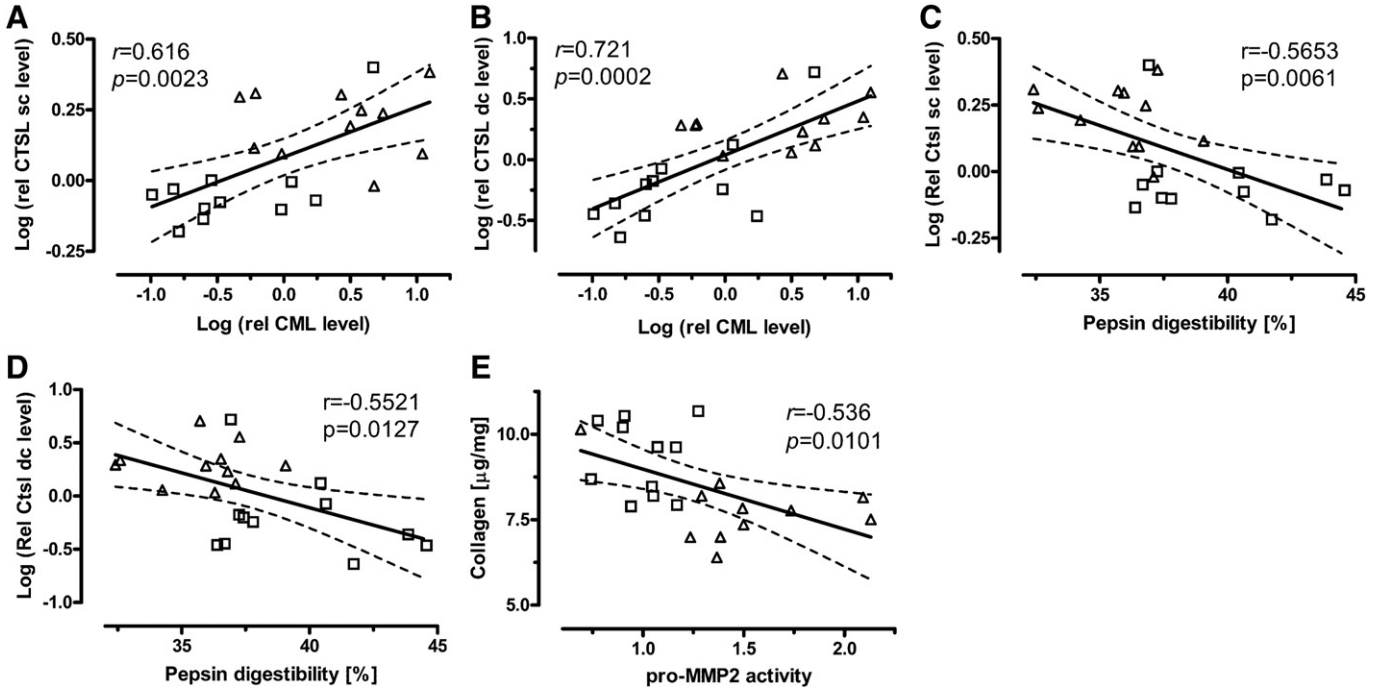

Fig. 8. Shown are correlation analyses of CTSL sc (A) and -dc (B) in kidney cortices *vs.* collagen-bound CML levels for placebo treated healthy- (white squares) and diabetic (white triangles) rats. Correlation analysis of CTSL sc (C) and -dc (D) in kidney cortices *vs.* pepsin digestibility of kidney collagen extracts is shown for the same group of rats. The correlation coefficient *r*, *P*-value and regression line with 95% confidence interval (dashed line) are shown in each graph: $n = 11/12$ per group.

a therapeutic option to lower glycoxidative stress in diabetes that could be incorporated as part of a therapeutic regimen in addition to glucose-lowering treatment.

Author contributions

Sebastian Brings conceived, designed and carried out the experiments, analysed and interpreted data and wrote the manuscript.

Shaoping Zhang conceived experiments, supervised research, interpreted data and revised the manuscript.

Bernard Choong designed and performed research.

Sebastian Hogl performed research and analysed data.

Martin Middleditch performed research and analysed data.

Deming Gong performed research.

Meder Kamalov designed and performed research.

Margaret A. Brimble conceived experiments and revised the manuscript.

Garth J. S. Cooper conceived and designed experiments, supervised research, interpreted data, and revised the manuscript.

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Conflict of interest statement

GJSC is named as inventor in patents that disclose the use of TETA for the treatment of diabetes. These patents are owned by PhilERA Inc., and he has no financial interest in them. All other authors declare no conflict of interest.

Transparency document

The Transparency Document associated with this article can be found, in the online version.

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